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Isolation, characterisation and antifungal activity of β-1,3-glucanase from seeds of *Jatropha curcas*

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A β-1,3-glucanase was isolated from *Jatropha curcas* using Sephadex G-75, SP Sepharose FF and HiPrepSephacrayS-100HR chromatography columns, successively. It exhibited a molecular weight (*Mr*) of 65-66kD consisting of three sub-units with non-covalent bond conjugate, as estimated using 8% (w/v) polyacrylamide gel electrophoresis (PAGE), 5–20% sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis (SDS-gradient PAGE) and gel filtration on a Sephadex G-75 column, respectively. The protein had an isoelectric point (*pI*) of 8.3 shown by isoelectric focusing (IEF). It exerted *in vitro* antifungal activity against *Rhizoctonia solani* Kuha. and *Gibberelle zeae* (Schw.) Petch. by hydrolysing cell walls of fungi. It was slightly toxic to mice LD_{50} 2.2g kg^{-1}. This protein may be a useful biological fungicide.

Abbreviations: DTT = dithiothreitol, FPLC = Fast Protein Liquid Chromatography, IEF = isoelectric focusing, *Mr* = molecular weight, PAGE = polyacrylamide gel electrophoresis, PDA = potato dextrose agar, PEG = polyethylene glycol, *pI* = isoelectric point, SDS-gradient PAGE = sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis

Introduction

Fungal pathogens are responsible for considerable yield loss in agriculture (Banzet *et al*. 2002). However, plants are always exposed to a large number of pathogenic fungi; although they do not have an immune system, plants have evolved a variety of potent defence mechanisms, including the synthesis of low-molecular-weight compounds, proteins and peptides that have antifungal activity (Claude 2001), of which β-1,3-glucanase is one of the most important antifungal proteins (AFPs) (Leah *et al.* 1991, Anuratha *et al.* 1996, Roulin *et al.* 1997, Mariaines *et al.* 2000). β-1,3-glucanases are present in many higher plants, and it is considered to be one component of a broad generalised defence mechanism against pathogen attack (Boller 1987, Collinge and Slusarenko 1987, Cornelissen and Melchers 1993). Considerable effort has thus been aimed at isolating these β-1,3-glucanases to evaluate their antifungal potential for improving resistance of plants against pathogenic fungi.

Wild *Jatropha curcas*, Euphorbiaceae, prefers light, warm and arid environments and survives in poor stoney soils. It has strong ability to resist pathogens (Chen and Zheng 1987). In the tropics, *J. curcas* is traditionally used for medicines and as hedges (Jones and Miller 1992), and the oil has been used for making soap (Keith 2000). *J. curcas* has economic importance in areas with extreme climates and soil conditions because of its extraordinary high drought resistance (Gübitz *et al.* 1997). *J. curcas* became a good candidate for forestation in dry hot valley regions, and the *J. curcas* industry was a key programme supported by the United Nations for helping the poor and protecting the environment. The seeds of *J. curcas* represent a good source of proteins, curcins, esterases and lipases (Stirpe *et al.* 1976, Huang *et al.* 1991, Barbieri *et al.* 1993, Staubmann *et al.* 1999, Lin *et al.* 2002). However, nobody screened for and purified β-1,3-glucanase from *J. curcas* seeds, generating our interest in *J. curcas*. To exploit the AFPs from *J. curcas*, β-1,3-glucanase were screened from seeds of *J. curcas*, and the *in vitro* antifungal activity was tested in our study. The present paper reports its isolation, characterisation and antifungal activity.

Materials and Methods

The seeds of *J. curcas* were collected from Panzhihua City, Sichuan Province, China. The plant fungal pathogens *Rhizoctonia solani* Kuha., *Sclerotinia sclerotiorum* (Lib) de Bary and *Gibberelle zeae* (Schw.) Petch. were provided by the Institute of Plant Protection, Chinese Agricultural Academy of Sciences, and maintained on PDA medium by

our laboratory. Sephadex G-75 (Pharmacia Co.) [2.6cm × 85cm chromatography column], SP Sepharose FF (Amersham Biosciences) [1.8cm × 20cm chromatography column] and HiPrep16/60 Sephacray S-100HR (Amersham Biosciences) were set up on a Fast Protein Liquid Chromatography (FPLC) system. All columns were washed and equilibrated with 5mM phosphate buffer containing 0.2M NaCl (pH 7.2). Molecular weight standards came from Watson Co. Other reagents were of analytical grade. All the assays were repeated three times.

Isolation procedure

The crude proteins of seeds were extracted according to the method of Lin *et al.* (2002). Six ml of crude protein solution was loaded on the Sephadex G-75 column, and the column was developed at a flow rate of 16ml h^{-1} until 110 4ml fractions were collected. β-1,3-glucanase activity peaks were collected. After concentration, using PEG-20 000, the protein mixtures were loaded on the column of the SP Sepharose FF. The column was washed using the same buffer. The activity peak, which was unadsorbed, was collected, concentrated and loaded on FPLC HiPrep16/60 Sephacray S-100HR column. The column was developed at a flow rate of 0.8ml min^{-1}. The purity of the eluted proteins with β-1,3-glucanase activity was determined.

SDS-gradient PAGE

SDS-gradient PAGE was performed according to the methods of Ansubel *et al.* (1998) and Guo (2001) to determine the purity of protein, numbers of its sub-units and its *Mr.* To determine how to link among sub-units, reduced protein sample [loading buffer containing Tris.Cl 100mmol l^{-1}, pH 6.8; Glycerol 20% (w/v); SDS 4% (w/v); dithiothreitol (DTT) 3% (w/v); Bromophenol blue 0.001% (w/v)] and non-reduced protein sample [loading buffer containing Tris.Cl 100mmol l^{-1}, pH 6.8; Glycerol 20% (w/v); SDS 4% (w/v); Bromophenol blue 0.001% (w/v)] were respectively loaded on the SDS-gradient PAGE with 5–20% separation gel after being heated at 100°C for 3min and running at 10mA (spacer gel) and 20mA (separation gel). The gel was stained by Coomassie brilliant blue.

PAGE

PAGE was carried out as described by Guo (2001). Eight micrograms of sample mixed loading buffer [Tris.Cl 100mmol l^{-1}, pH 6.8; Glycerol 20% (w/v); Bromophenol blue 0.001% (w/v)] was loaded on PAGE with 8% acrylamide gel, running at 10mA (spacer gel) and 20mA (separation gel). The gel was stained by Coomassie brilliant blue.

***Apparent* Mr**

The apparent *Mr* of the purified β-1,3-glucanase was determined by gel filtration as described by Zhang *et al.* (1997). A molecular sieve Sephadex G-75 chromatography column was calibrated with molecular weight markers (cytochrome c 11.7kD, myoglobin 17.2kD, chymotryp-sinogen 25.7kD, ovalbumin 43.0kD, bovine serum albumin 67.0kD). The regression curve was obtained when 1g *Mr* values were used as abscissa, and Kav. values were used as ordinate. The regression equation was formed from software Origin 6.0. *Mr* was calculated according to the regression equation.

IEF

Isoelectric focussing was performed by the method of Ansubel *et al*. (1998) in 1.5mm diameter disc tubes with 8% polyacrylamide gel containing 2% (v/v) ampholyte (pH 4–10), using the Bio-Rad's IEF standards, a mixture of nine natural proteins with *pI* values of 4.45 to 9.6. The sample and the IEF standards were respectively loaded on different disc tubes. The voltage was increased stepwise: 200V for 1h and 800V for 16h. The gel was stained by Coomassie brilliant blue.

β-1,3-glucanase activity

The enzyme assay was performed according to the method of Wang *et al.* (1992) with some modifications in 1.5ml solution containing 0.6% (mass vol^{-1}) laminaran (Sigma), 50mM sodium acetate (pH 5.5) and 0.3234mg ml^{-1} of purified protein. The activity was determined by measuring the content of glucose every 10min according to the method of Somogyi (1952).

Antifungal activity

Antifungal activity against *R. solani* Kuha., *S. sclerotiorum* (Lib) de Bary and *G. zeae* (Schw.) Petch. was estimated as described by Dash *et al*. (2001) and Lam and Ng (2001).

For hyphal growth inhibitory assay, freshly-grown fungal mycelium was spot-inoculated at the centre of a Petri dish containing potato dextrose agar (PDA) medium and inoculated at 28°C for 24h. Holes (5mm in diameter) were bored into the agar with a gel punch in front of the growing fungal mycelium, and 3.15nM, 6.3nM and 12.6nM protein samples were respectively pipetted into the wells. Buffer only without β-1,3-glucanase served as a negative control. The plates were examined for zones of inhibition after 24h incubation at 28°C.

For the spore formation inhibitory assay and a quantitative assay to determine the IC_{50} of antifungal activity, 5nM, 12.6nM and 15.8nM protein samples were added into 10ml PDA medium at 45°C respectively, mixed rapidly and poured into small Petri dishes. Buffer only without β-1,3-glucanase served as a negative control. After the agar had cooled down, a small amount of hyphae was inoculated. After incubating at 28°C for 48h, the cultures were examined under microscope for formation of spores, and the diameters of the hyphae colonies were measured for IC_{50} value.

For a more detailed microscopic examination, the hyphae growing on PDA medium containing β-1,3-glucanase were collected to be investigated using a transmission electron microscope.

Toxicity test in mice

Toxicity was evaluated in mice weighing 20–25g. The purified proteins were injected intraperitoneally into groups of five animals per dose. Buffer only without antifungal protein served

as a negative control. The LD_{50} was calculated by regression equation according to the method of Molinengo (1979) and Meier and Theakstoon (1986) (95% confidence limits).

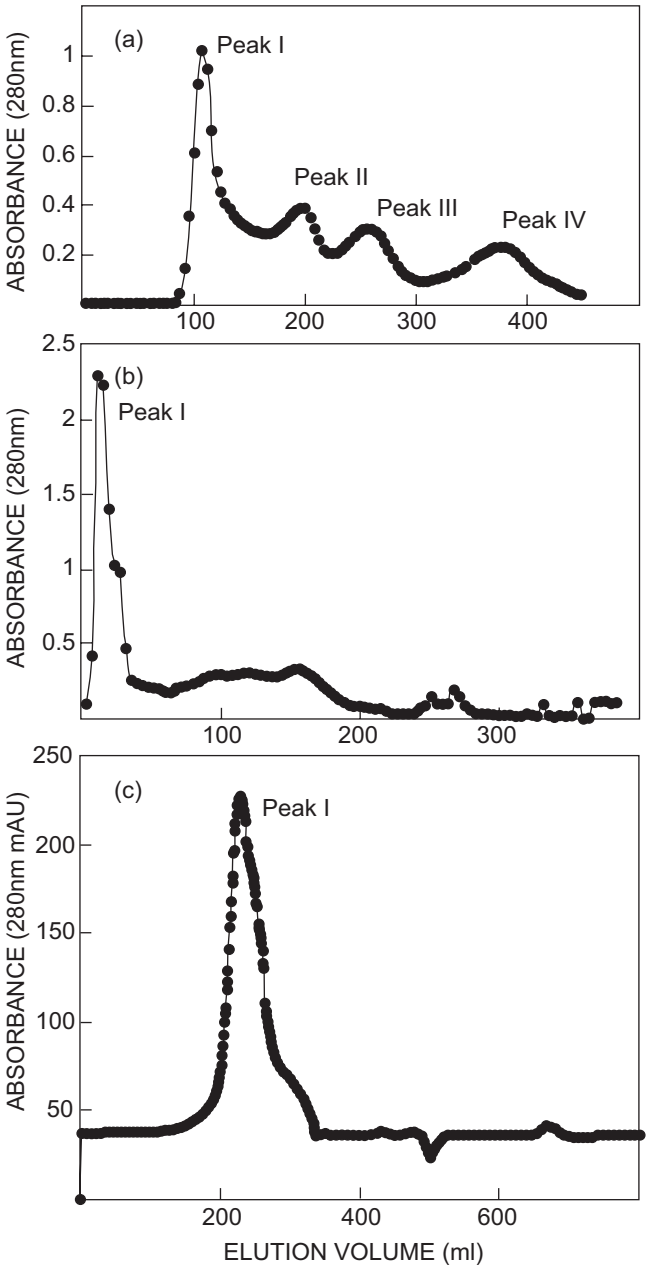

Figure 1: Purification of β-1,3-glucanase. (A) Elution profile of the crude protein of *J. curcas* seeds from molecular sieve Sephadex G-75 chromatography column. Peak I showed β-1,3-glucanase activity; (B) Cation exchange chromatography on SP Sepharose FF from peak I of Figure 1A. Peak I showed β-1,3-glucanase activity; (C) HiPrep 16/60 Sephacryl S-100HR chromatography on FPLC from peak I of Figure 1B. Peak I showed β-1,3-glucanase activity

Results

Purification and characterisation

Molecular sieve chromatography of the crude proteins of *J. curcas* seeds through Sephadex G-75 column yielded four major peaks (I, II, III and IV) (Figure1A). Peak I had β-1,3-glucanase activity. The ion-exchange chromatography of peak I from Sephadex G-75 through SP Sepharose FF yielded a large unadsorbed peak (peak I) which had β-1,3-glucanase activity and a few small adsorbed peaks without β-1,3-glucanase activity (Figure 1B). Peak I from SP Sepharose FF chromatography was analysed by FPLC on a HiPrep16/60 Sephacray S-100HR column. The β-1,3-glucanase activity of peak I (Figure 1C) of FPLC was confirmed, and the front half of the peak I exhibited the *Mr* of approximately 65kD in PAGE (Figure 2B) and 66kD in molecular sieve chromatography on Sephadex G-75 (data not shown). It exhibited three bands with *Mr*s of 20kD, 22kD and 23kD in SDS-gradient PAGE successively, under reducing condition (Figure 2A, lane 1) or non-reducing condition (Figure 2A, lane 2). The *pI* of the protein was 8.3 by IEF analysis (data not shown). But the rest of peak I from the HiPrep16/60 Sephacray S-100HR column showed some minor bands except the three major bands of 20kD, 22kD and 23kD. It was a mixture.

β-1,3-glucanase activity and antifungal activity

After mixing laminaran and the purified protein, the content of linear increased glucose was determined in 50min from a mixture containing the purified protein. The specific activity of β-1,3-glucanase was 181nmol mg^{-1} min^{-1} (data not shown).

Three phytopathogenic fungi were used in the assay, and the protein exerted varying antifungal activity against the different pathogens. It showed an inhibitory effect on hyphal extension of *R. solani* Kuha. (Figure 3A). The IC_{50} values were found to be 12.6nM (Figure 3B). There was almost no spore formation of *R. solani* Kuha. in both the control and hyphae treated by β-1,3-glucanase. Hyphal growth of *G. zeae* (Schw.)

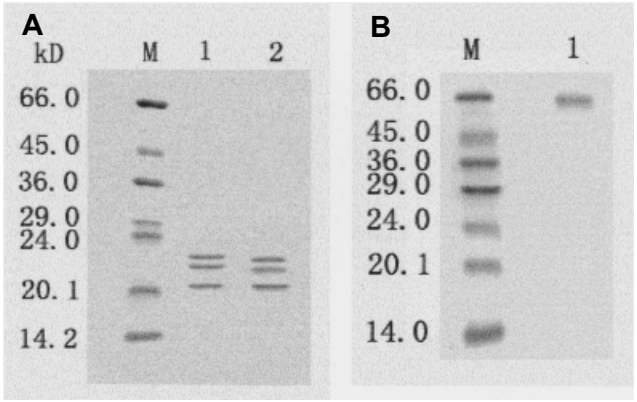

Figure 2: SDS-gradient PAGE and PAGE of β-1,3-glucanase. (A) SDS-gradient PAGE: M: Marker, lane 1: β-1,3-glucanase (sample buffer containing DTT), lane 2: β-1,3-glucanase (without DTT); (B) PAGE: M: Marker, lane 1: β-1,3-glucanase

Petch. was also inhibited by the protein, but appeared to be less sensitive than *R. solani* Kuha. The obvious change was the colour of *G. zeae* (Schw.) Petch, from red to yellow (Figure 3C). The colour change of the hyphae from red to yellow indicated an inhibition of conidia (Zeng *et al.* 1997). Purified protein had no effect on the hyphal growth of *S. sclerotiorum* (Lib) de Bary.

A more detailed microscopic examination of the effect of the protein on *R. solani* Kuha. and *G. zeae* (Schw.) Petch. was performed. Under transmission electron microscope, the common effect of the β-1,3-glucanase on hyphal growth inhibition could be detected in the presence of cell wall. The cell walls of hyphae treated by β-1,3-glucanase became thin and weak (Figure 4).

Toxicity test in mice

The protein was toxic to mice at high concentrations. Figure 5 shows the survival dosage (D) and the rate of survival dosage (D) / Time (T), of mice. When given 1.6g kg^{-1} of the protein, the mice did not die after a week. At a dose of 2.12g kg^{-1}, poisoning symptoms began after 20h and deaths mostly occurred after 22h. All mice died within 6h with a dose of 4.17g kg^{-1}. The calculated LD_{50} in mice was about 2.2 ± 0.52g kg^{-1} (95% confidence limits). It showed low toxicity.

Discussion

This paper is the first reported purification of β-1,3-glucanase from *J. curcas* seeds. The result of SDS-gradient PAGE in reducing conditions (loading buffer containing DTT), three bands with *Mr*s of 20kD, 22kD and 23kD (Figure 2A, lane 1), was the same as that in non-reducing conditions (without DTT in loading buffer) (Figure 2A, lane 2). The calculated *Mr*s of β-1,3-glucanase by both PAGE (Figure 2B, lane 1) and gel filtration on Sephadex G-75 column (data not shown) were 65-66kD. It corresponded to the sum of *Mr*s of 20kD, 22kD and 23kD. We propose that β-1,3-glucanase should have *Mr* of 65–66kD and consist of three sub-units with non-covalent bond conjugate (Wang *et al.* 2002), and the *Mr*s of three subunits were 20kD, 22kD and 23kD. However, further study on the three sub-units, such as the amino acid composition, N-terminal sequence analysis, etc., is needed.

The antifungal properties of β-1,3-glucanase in *J. curcas* showed that it not only inhibited the growth of hyphae (Figure

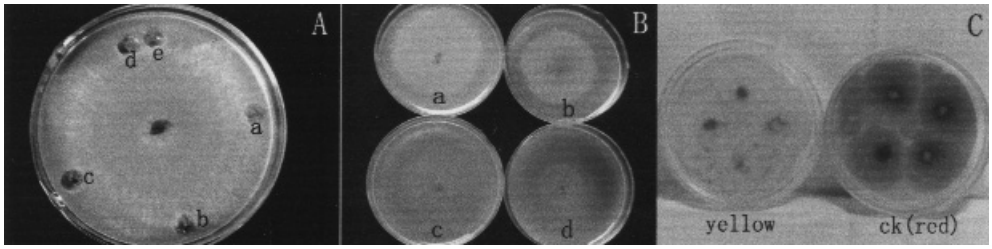

Figure 3: Inhibitory activity of β-1,3-glucanase toward *R. solani* Kuha. and *G. zeae* (Schw.) Petch. (A) Inhibitory effect of β-1,3-glucanase toward *R. solani* Kuha., (a) 100μl of buffer as negative control, (b) 3.15nM protein, (c) 6.3nM protein, (d)+(e) 12.6nM protein; (B) Determination of the IC_{50} value of antifungal activity of β-1,3-glucanase toward *R. solani* Kuha., Plate (a) represented the control (only buffer without β-1,3-glucanase) and plates (b), (c) and (d) represented treatments with 5nM, 12.6nM, 15.8nM β-1,3-glucanase, respectively. The IC_{50} was calculated to be 12.6nM; (C) β-1,3-glucanase made the colour of hyphae of *G. zeae* (Schw.) change from red to yellow. Ck is the control

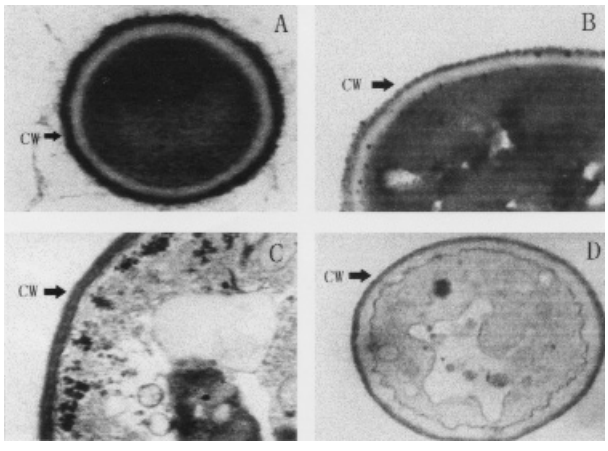

Figure 4: The mycelial cell wall under transmission electron microscope: (A) Mycelial cell wall of *R. solani* Kuha. (control) was thick; (B) Mycelial cell wall of received treatment *R. solani* Kuha. was thin; (C) Mycelial cell wall of *G. zeae* (Schw.) Petch. (control) was thick; (D) Mycelial cell wall of received treatment *G. zeae* (Schw.) Petch. was thin

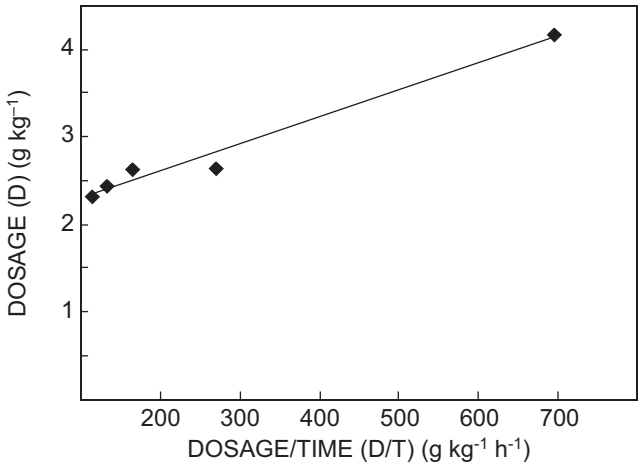

Figure 5: The determination of LD_{50} in mice. It shows the LD_{50} was 2.23 ± 0.52g kg^{-1} h^{-1} (95% confidence limits)

3A), but also inhibited spore formation (Figure 3C). These findings might be very important for overexpressing β-1,3-glucanase in transgenic plants to enhance resistance against fungal pathogens (De Bolle *et al.* 1996). However, the inhibitory tests of β-1,3-glucanase on other pathogenic fungi are required for more wide-ranging application in agriculture. This study is being performed now.

The reason for the cell walls of hyphae treated with β-1,3-glucanases becoming thin and weak (Figure 4) may be because PR-2 proteins hydrolyse the structural β-1,3-glucan present in the fungal cell wall, or it may inhibit the synthesis of the fungal cell wall or disrupt cell wall structure (Claude 2001). β-1,3-glucan is present in many fungal cell walls, but usually only in small quantities in higher plants, so when plants are exposed to a large number of pathogenic fungi, β-1,3-glucanase may be induced (Boller 1987, Du and Wu 1990, Ai 1995). The fungal cell wall provides an experimental target for antifungal antibiotics — β-1,3-glucanase and the plant cell wall was not affected (Claude 2001).

In summary, a three sub-unit protein with antifungal activity and β-1,3-glucanase activity was purified from *J. curcas* seeds in this study. The low mammal toxicity and strong inhibition against fungal pathogens suggest that the protein could be of use to control fungal pathogens.

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